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Specific HDV RNA-templated transcription by pol II in vitro

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ABSTRACT

RNA polymerase II is implicated in the RNA-templated RNA synthesis during replication of viroids and Hepatitis Delta Virus (HDV); however, neither the RNA template nor protein factor requirements for this process are well defined. We have developed an in vitro transcription system based on HeLa cell nuclear extract (NE), in which a segment of antigenomic RNA corresponding to the left-hand tip region of the HDV rod-like structure serves as a template for efficient and highly specific RNA synthesis. Accumulation of the unique RNA product is highly sensitive to α -amanitin in HeLa NE and only partially sensitive to this drug in NE from PMG cells that contain an allele of the α -amanitin-resistant subunit of pol II, strongly suggesting pol II involvement in this reaction. Detailed analysis of the RNA product revealed that it represents a chimeric molecule composed of a newly synthesized transcript covalently attached to the 5' half of the RNA template. Selection of the start site for transcription is remarkably specific and depends on the secondary structure of the RNA template, rather than on its primary sequence. Some features of this reaction resemble the RNA cleavage-extension process observed for pol II-arrested complexes in vitro. A possible involvement of the described reaction in HDV replication is discussed.

Keywords: HDV (Hepatitis Delta Virus); pol II; RNA replication; RNA-templated transcription; viroids

INTRODUCTION

RNA synthesis in eukaryotes is carried out by RNA polymerases I, II, and III (Roeder, 1976). Each polymerase transcribes functionally distinct DNA sequences controlled by specific promoter elements. In particular, RNA polymerase II (pol II) responsible for transcription of pre-mRNAs has been extensively studied both in terms of DNA template requirements and protein factors that regulate its activity. Pol II has also been implicated in replication of two classes of RNA pathogens: plant viroids and human Hepatitis Delta Virus (HDV) (Robertson & Branch, 1987; Taylor, 1992; Lai, 1995). Viroids are small (~250–600 nt), single-stranded, circular RNAs that can be folded into unbranched rod-like structures (Keese & Symons, 1987). They replicate in plant cells by a rolling circle mechanism, in which a circular template of one polarity is used to generate multimeric intermediates of the opposite polarity. These multimeric RNAs are self-cleaved by ribozyme elements encoded in the viroid sequence, yielding monomeric RNAs that are subsequently ligated, possibly by

a host RNA ligase activity, to generate circular products (Branch & Robertson, 1984; Robertson & Branch, 1987).

Similarly, HDV RNA, a satellite of Hepatitis B Virus (HBV), is a 1.7-kb single-stranded circular RNA that, like viroids, can be folded into an unbranched rod-like structure and is thought to replicate by a rolling circle mechanism (Taylor, 1992, and references therein; Lai, 1995). Both polarities of HDV RNA [genomic (G) and antigenomic (AG)] contain HDV ribozyme domains (Fig. 1A) that are responsible for processing of multimeric intermediates to monomeric products (Taylor, 1992, and references therein; Lai, 1995). One significant difference between viroids and HDV concerns their coding potential. Although viroids do not code for any polypeptides, HDV RNA encodes a single protein, Hepatitis Delta Antigen (HDAg). Although HDAg is essential for replication of HDV, it is not a replicase itself (Kuo et al., 1989; MacNaughton et al., 1991). Furthermore, HBV helper virus does not provide replicase function either, as HDV cDNA- or RNA-transfected cell lines efficiently support HDV RNA replication in the absence of any HBV proteins (Kuo et al., 1989; Glenn et al., 1990). Thus, while viroids and HDV must fully depend on the host cell machinery for their replication, the identity of the polymerase activity involved has not been demonstrated.

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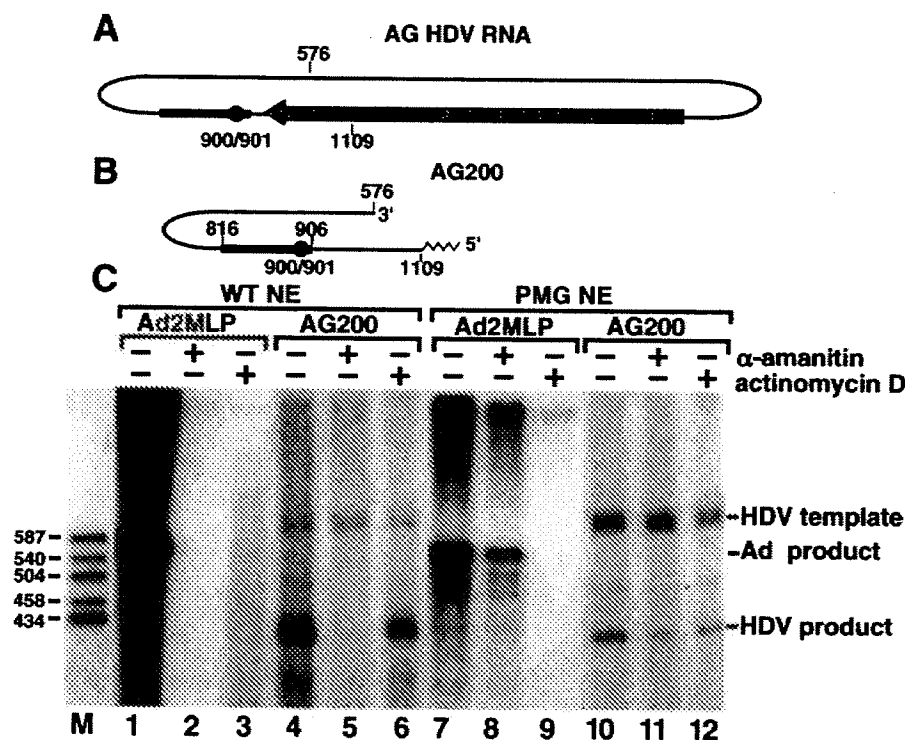


FIGURE 1. Antigenomic (AG) HDV RNA serves as a template for RNA synthesis by pol II. **A:** Schematic representation of the full-length single-stranded circular AG HDV RNA folded into an unbranched rod-like structure. The AG ribozyme domain (rectangle) and its site of cleavage (circle) are indicated. The arrow depicts the open reading frame for HDVAg. **B:** AG200, the segment of AG HDV RNA used as a template in NE transcription reactions. **C:** RNA synthesis in wt (lanes 1–6) and PMG (lanes 7–12) HeLa NE using Ad2MLP DNA (lanes 1–3 and 7–9) or HDV RNA (lanes 4–6 and 10–12) as templates. Transcription reactions were monitored by incorporation of α - 32 P-GTP into newly synthesized RNAs were carried out under standard conditions (lanes 1, 4, 7, and 10), in the presence of 1 μ g/mL α -amanitin (lanes 2, 5, 8, and 11), or 20 μ g/mL actinomycin D (lanes 3, 6, 9, and 12). Products were resolved in a 5% polyacrylamide/8 M urea gel. Lane M contains pBR322xHaeIII DNA size marker. Positions of Ad2MLP and HDV RNA transcription products and HDV RNA templates end labeled in the presence of NE are indicated.

Synthesis of viroid RNAs has been studied in vitro using intact protoplasts or cell free nuclear homogenates of different plant cells in the presence of inhibitors of specific RNA polymerases. Inhibition of viroid synthesis by α -amanitin at concentrations known to inhibit pol II suggested the involvement of this activity in viroid replication (Mühlbach & Sanger, 1979; Rackwitz et al., 1981; Flores & Semancik, 1982; Semancik & Harper, 1984; Spiesmacher et al., 1985; Rivera-Bustamante & Semancik, 1989). However, *Escherichia coli* RNA polymerase, *E. coli* DNA polymerase I, Q- β replicase, and RNA-dependent RNA polymerase activity from tomato are also able to copy viroid RNA in vitro (Owens & Diener, 1977; Boege et al., 1982; Rohde et al., 1982) in the presence of Mn^{2+} ions, conditions known to reduce the template specificity of RNA polymerases. Pol II has also been implicated in HDV replication. HDV RNA synthesis in assays using isolated nuclei or nuclear homogenates from hepatoma cell lines is sensitive to α -amanitin (MacNaughton et al., 1991; Fu & Taylor, 1993). Although these experiments are highly suggestive of pol II involvement, details concerning specificity of transcription initiation, protein factors, and RNA template requirements for this process remain to be established.

Here we describe an in vitro system based on HeLa cell nuclear extracts (NE), in which a specific segment of AG HDV RNA serves as a template for efficient and

highly specific RNA synthesis. This RNA-dependent RNA synthesis involves pol II, as indicated by α -amanitin sensitivity of the process. The pol II involvement is further documented by partial α -amanitin resistance of the RNA synthesis in PMG cell extracts that contain an α -amanitin resistant allele of the largest subunit of pol II (Bartolomei & Corden, 1987; J.L. Corden, pers. comm.). The RNA product generated in these reactions represents a chimeric molecule composed of the newly transcribed RNA covalently attached to the 5' portion of the template. Selection of the start site for transcription is remarkably specific and determined by the secondary structure of the RNA template, rather than by its primary sequence. This mode of RNA synthesis is reminiscent of the RNA cleavage-extension reactions observed in vitro for RNA polymerase-arrested complexes (reviewed by Uptain et al., 1997).

RESULTS

Pol II in HeLa cell NE utilizes segments of AG HDV RNA as templates for transcription

To study the template and protein factor requirements for RNA-dependent RNA synthesis, we have developed a system in vitro in which segments of HDV RNA are used as templates for transcription by pol II in NE of HeLa cells. The pol II activity of these extracts was

confirmed in standard reactions using Adenovirus 2 major late promoter DNA (Ad2MLP) as a template. As expected, accurate initiation of transcription from this DNA generated a 536-nt RNA product (Fig. 1C, lane 1). RNA synthesis in these reactions is completely inhibited by α -amanitin (1 μ g/mL) (Fig. 1C, lane 2), consistent with the involvement of pol II, and by actinomycin D (20 μ g/mL) (Fig. 1C, lane 3), consistent with the use of a DNA template in this process.

Several segments of genomic and antigenomic polarity HDV RNA were tested as templates for RNA synthesis in this system. Most of them become end labeled in these reactions (e.g., see slower migrating bands in Fig. 1C, lanes 4–6 and 10–12), as demonstrated by RNase H analysis in the presence of complementary DNA oligonucleotides (data not shown). One of the templates, AG200 RNA (Fig. 1B), yielded in addition a unique, faster migrating product (Fig. 1C, lane 4). The AG200 RNA template contains 533 nt of AG HDV RNA from positions 576 to 1109 (Wang et al., 1986), flanked at its 5' end by a 59-nt polylinker sequence. Synthesis of ~400-nt-long product is only modestly inhibited in the presence of actinomycin D (Fig. 1C, lane 6), consistent with the RNA-, rather than DNA-templated process, but is strongly inhibited by α -amanitin (Fig. 1C, lane 5), suggesting pol II involvement. In contrast, template labeling is not significantly affected by either of these toxins (Fig. 1C, lanes 5 and 6). To confirm pol II involvement in the RNA-templated transcription reaction, NE was prepared from PMG HeLa cell line that contains a copy of the mouse RPII215 gene encoding an α -amanitin resistant variant of the largest subunit of pol II (Bartolomei & Corden, 1987; J. Corden, pers. comm.). In control experiments using the Ad2MLP DNA template, transcription in PMG NE was reduced approximately threefold in the presence of α -amanitin (Fig. 1C, lanes 7 and 8). This result was expected, because in addition to the integrated mutant gene, PMG cells still contain the endogenous α -amanitin-sensitive pol II alleles. RNA-templated transcription in the PMG NE was also reduced two- to threefold (Fig. 1C, lanes 10 and 11), as in the case of the DNA-templated reaction (Fig. 1C, lanes 7 and 8). These results strongly suggest that pol II in the HeLa NE can use HDV RNA as a template. However, additional NE components must also be required, since a purified pol II preparation (gift of A. Hoffmann and R.G. Roeder) did not support RNA transcription under these conditions (data not shown). Further studies will be required to determine if any of the known protein components of pol II machinery are involved in this RNA-templated process.

Minimal HDV RNA template for pol II in HeLa cell NE

To identify the minimal segment of AG HDV RNA that serves as a template for pol II in HeLa NE, we per-

formed a deletion analysis of the initial AG200 template. Progressive deletions of HDV sequences from either end of the template do not significantly affect the efficiency of transcription. In fact, a 280-nt AG128 RNA (Fig. 2A) is used even more efficiently than the 592-nt AG200 RNA template (Fig. 2B, lane 1; data not shown). A number of RNAs containing internal deletions—AG130, AG131, AG129, and AG103 (see Fig. 2A)—serve as efficient templates *in vitro* (Fig. 2B, lanes 2, 3, 6, and 7, respectively) whereas efficiency of RNA synthesis using other templates, for example, AG132 and AG138 RNA, is greatly diminished (Fig. 2B, lanes 4 and 5). Interestingly, in cases where ~30-nt deletions on either side of the terminal hairpin disrupt the overall unbranched rod-like structure (as predicted by computer modeling), the efficiency of transcription is strongly inhibited (AG132 and AG138, Fig. 2B, lanes 4 and 5). A combination of these two deletions in AG103 restores the unbranched hairpin structure and results in efficient transcription (Fig. 2B, lane 7). These results suggest that the characteristic structure of the RNA template in proximity of the hairpin tip is important for efficient transcription. Similar ~30-nt deletions introduced more distally (AG130 and AG131) do not have a significant effect (Fig. 2B, lanes 2 and 3).

RNA synthesis using all these templates is sensitive to α -amanitin, consistent with the involvement of pol II in these reactions. The apparent sizes of generated RNA products provided an initial information concerning the start site of transcription. As expected, templates with deletions in the 5' half of the molecule, for example, AG130 and AG138 (Fig. 2B, lanes 2 and 5, respectively), yielded products that migrated faster than that of AG128 RNA, and the change in product mobility correlated well with the size of the introduced deletion (~30 nt). However, deletions in the 3' portion of the template did not affect gel mobility of the corresponding products (compare AG128 with AG131 and AG132, Fig. 2B, lanes 1, 3, and 4). These results suggest that the start site of the observed transcription is located upstream (5') of the region deleted in AG132 RNA and downstream (3') of the region deleted in AG138 RNA, that is, near the loop of the hairpin template. Consistent with this notion, double deletions in both 5' and 3' regions of the template (e.g., AG129 and AG103, Fig. 2B, lanes 6 and 7) affect the mobility of products to the same extent as single deletions in the 5' region only (e.g., AG130 and AG138, Fig. 2B, lanes 2 and 5). Further deletions of the 218-nt AG103 RNA from either the 5' or 3' end inactivated these templates (data not shown), and thus AG103 RNA was used as a standard, minimal template in all subsequent experiments.

Transcription on AG103 RNA template is fast and efficient. Full-length product (P) is detectable within 5 min of incubation and continues to accumulate for up to an hour (Fig. 3). The full-length labeled template (T) that can also be detected within 5 min of incubation is par-

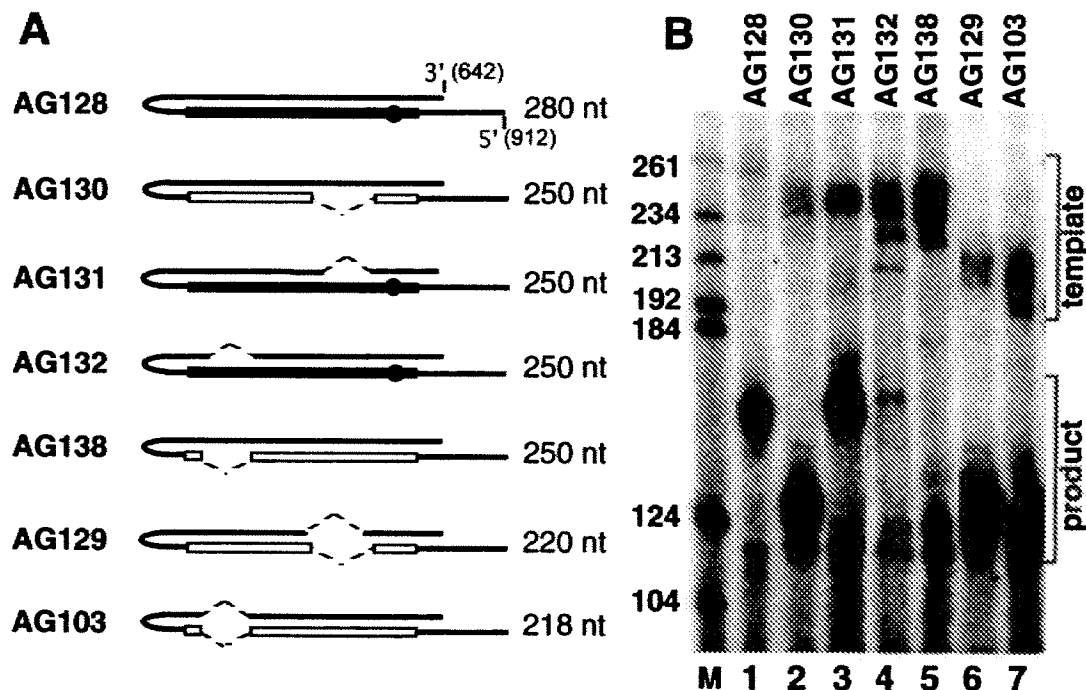


FIGURE 2. Deletion analysis of HDV RNA segment required as a template for NE transcription. **A:** Schematic representation of RNA templates. AG128 RNA contains HDV sequence (positions 912–642) spanning the left-hand terminal portion of the rod-like structure and includes the AG ribozyme domain. Subsequent internal deletions of this template generated AG130 (Δ positions 863–890), AG131 (Δ positions 693–718), AG132 (Δ positions 718–768), and AG138 (Δ positions 822–858). To generate AG129 and AG103, deletions of AG130+AG131 and AG132+AG138, respectively, were combined. Positions of the cleavage site (circle) of AG ribozyme (rectangle) are indicated. Active ribozymes are represented by closed symbols, whereas those inactivated by the introduced deletions are shown as opened rectangles. **B:** Transcription reactions using templates represented in **A**. In addition to the labeled NE RNA products (~125–155 nt), some end labeling of the full-length RNA templates was frequently observed. Products were resolved in a 5% polyacrylamide/8 M urea gel. Positions of pBR322xHaeIII DNA marker are indicated (lane M).

tially degraded (t) during the time course of incubation; however, its overall level remains constant. Although the full-length product formation requires the presence of all four NTPs, the template RNA becomes efficiently labeled even upon addition of any one of ^{32}P - α -NTP alone (data not shown). Together, these results suggest that NE RNA product is not generated by a simple processing of the labeled template. Consistent with the α -amanitin results (Fig. 1C), optimal conditions for NE product formation with regard to temperature, Mg^{2+} , K^+ , and NE concentrations fall into ranges typical for pol II transcription in DNA-templated *in vitro* systems (see Material and Methods).

Sequence analysis of RNA products of the HDV RNA-templated reactions

To determine the precise start site of pol II transcription on the HDV RNA template *in vitro* and to evaluate the fidelity of this process, a detailed RNA sequence analysis was performed on the product of AG103 RNA-templated reaction (NE103 RNA). Gel-purified NE103 RNA product was subjected to exhaustive digestion

with RNase T1 (cleaves at G \downarrow) or RNase A (cleaves at Py \downarrow), and resulting oligonucleotides were resolved in a 25% polyacrylamide/8 M urea gel. In parallel, synthetic genomic polarity G HDV RNAs complementary to either the entire AG template (e.g., T3-G103-77, T3-G129) or only its 5' half (SP6-G103; Fig. 4B) were digested with RNases and used as oligonucleotide markers. The predicted RNase T1 oligonucleotides for SP6-G103 are shown in Figure 4B and the actual RNase T1 digestion pattern in Figure 5A, lane 1.

RNase T1 digestion of NE103 RNA product (Fig. 5A, lane 3) generates a subset of oligonucleotides in common with the SP6-G103 RNA digestion pattern. All of the labeled fragments in NE103 RNA digestion correspond to a region of ~40 nt of SP6-G103 RNA (Fig. 4B, shaded area), suggesting that only a portion of the AG template is copied in this reaction. To better characterize the RNA product, a number of mutant templates were analyzed in parallel. Sequence changes introduced into these templates result in characteristic patterns of the corresponding NE RNA products, confirming the identity of the copied region. For example, RNase T1 digestion of NE129 RNA product generates a set of

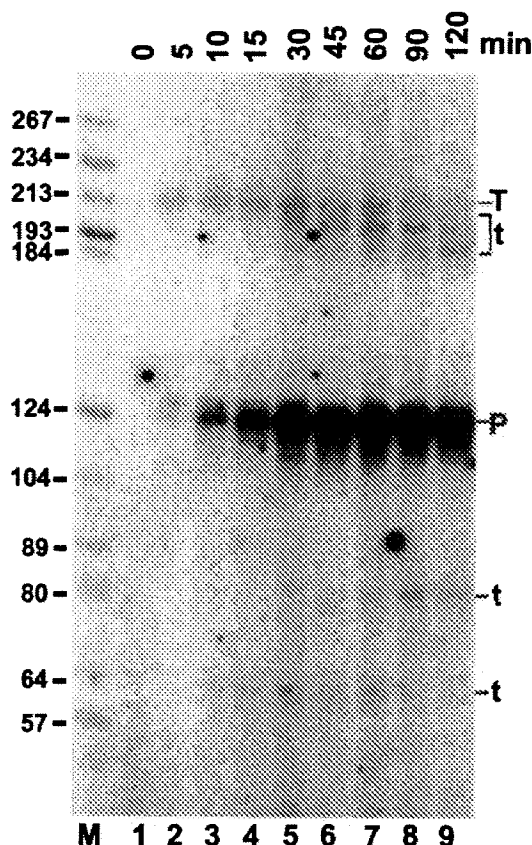


FIGURE 3. The time course of AG HDV RNA-templated transcription in the HeLa cell NE. Transcription reaction was incubated at 30°C and aliquots were withdrawn at the times indicated. RNA products were phenol extracted and resolved in a 5% polyacrylamide/8 M urea gel. Positions of the labeled template (full-length: T, and its degradation fragments: t), NE RNA product (P), and pBR322xHaeIII DNA markers (M) are indicated.

fragments distinct from those detected in NE103 RNA (see Fig. 4C for sequences). Specifically, sequence differences between AG129 and AG103 RNAs explain the observed lack of a 4-nt fragment in NE129 RNA and changes of 11 → 10 nt and 7 → 8 nt fragments (Fig. 5A, compare lanes 2 and 3; see Fig. 4B,C). Similarly, digestion of NE103-77 RNA product yields the expected 7-nt fragment that replaces an 8-nt oligo found in NE103 RNA (Fig. 5A, lane 6). In contrast, RNase T1 patterns of NE103-12 and 103-16 RNA products are not changed (Fig. 5A, lanes 4 and 5, respectively), as the corresponding mutations in their RNA templates do not affect any of the characteristic RNase T1 oligonucleotides (Fig. 4C). The detailed RNase digestion analysis of NE RNA products generated from a number of mutant templates demonstrates that they contain a portion of G polarity RNA precisely copied from AG RNA templates (Fig. 5A,B, and data not shown).

The most 3' oligonucleotide identified in the newly synthesized portion of NE103 product is RNase T1 4-mer ACCG (Fig. 5A, lane 3; see also the shaded area

in Fig. 4B). Genomic polarity sequence immediately downstream of this 4-mer contains a characteristic RNase A 8-mer GAGGAGGU; however, no fragment of this size is detected among RNase A digestion products of NE103 RNA (data not shown). Furthermore, the G → A mutation in the ACCG 4-mer does not change the RNase T1 digestion pattern (data not shown), suggesting that polymerase must terminate synthesis directly 3' of the ACCG sequence.

In all NE RNA products, the 5-nt AAUCG represents the 5'-most ³²P-labeled RNase T1 oligonucleotide of the transcript. To determine if any nucleotides upstream of this 5-mer are also transcribed, two additional templates, AG103-63 and 103-75, were prepared, in which sequences in the hairpin loop region encompassing the AAUCG 5-mer are mutated (see Fig. 4C for sequences). If transcription initiates upstream of this 5-mer, the introduced mutations will change the RNase T1 pattern of NE RNA product, defining the site of initiation. In digestions of NE103-63 RNA, the AAUCG 5-mer is replaced by a new 12-nt fragment (Fig. 5B, lane 4), indicating that the RNA product contains at least 7 nt of an upstream sequence. Similarly, in digestions of NE103-75 RNA, a new 20-nt fragment appears instead of AAUCG and AAAAG 5-mers (Fig. 5B, lane 7), consistent with the introduced mutations of RNase T1 sites located between these two fragments (Fig. 4C). The 5'-most RNase T1 fragments of NE103-63 and 103-75 products (12 nt and 20 nt, respectively; Fig. 5B, lanes 4–9), are 1 nt shorter than the corresponding 5'-terminal oligos in G polarity RNAs (Fig. 5B, lanes 10 and 11), indicating that they span the initiation site of the newly transcribed RNAs.

Typically, newly transcribed RNAs contain either a 5'-terminal triphosphate group (pppN) or a cap structure (m7GpppN). However, phosphatase or β-elimination/phosphatase treatments of NE RNA products, expected to remove possible 5' pppN or m7GpppN structures, respectively, do not affect electrophoretic mobility of any of the RNase T1 fragments (Fig. 5B, lanes 1–9). Instead, gel-purified 12-nt and 20-nt fragments derived from NE103-63 and 103-75 RNAs, respectively, that encompass the 5' end of the newly transcribed RNA contain 5'-OH groups, as they can be phosphorylated by T4 polynucleotide kinase (data not shown). Such a feature is typical of oligonucleotides excised by RNase from a longer RNA molecule. However, the copied region accounted for by the RNase digestion corresponds to only an ~40-nt sequence, whereas NE103 RNA product migrates as an ~125-nt RNA. Taken together, these results suggest that transcription in this reaction does not proceed to the 5' end of the AG template and the newly transcribed sequence is covalently attached to a longer RNA, perhaps the AG template itself.

To verify that the generated RNA product contains a portion of AG RNA template, gel purified NE103 RNA

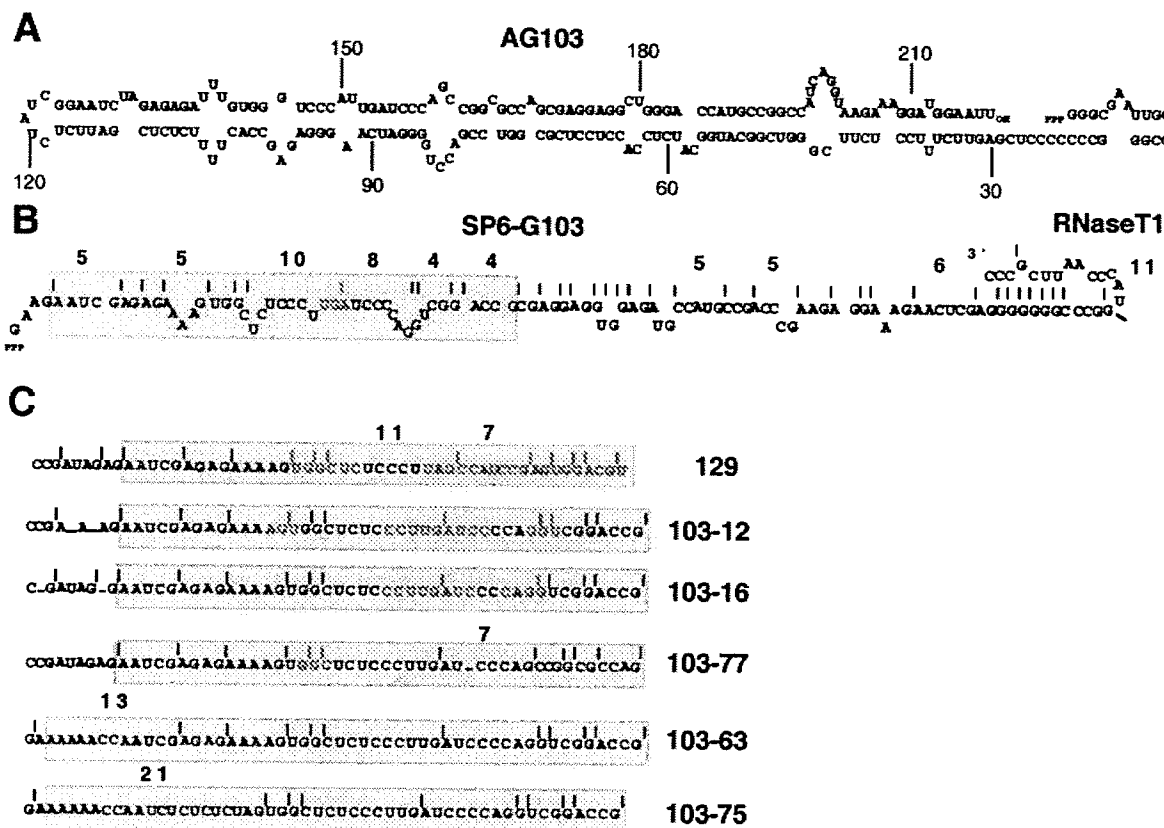


FIGURE 4. Sequences of AG and G HDV RNA segments with annotated RNase T1 digestion sites. **A:** Sequence of the minimal AG103 HDV RNA template. The numbering system corresponds to nucleotide positions in the RNA template. **B:** SP6-G103 represents a genomic (G) polarity RNA containing a copy of the bottom strand of the AG103 template, except for 2 nt at its 5' end introduced by the SP6 primer. RNase T1 cleavage sites are represented by vertical bars. The length (in nucleotides) of characteristic RNase T1 oligonucleotides used as size markers are indicated. Boxed regions represent segments of G RNA detected in NE RNA products (see Fig. 4A,B). **C:** G polarity sequences of mutant templates analyzed in **A** and **B**. RNase T1 digestion sites are annotated as in Figure 3B, but only oligonucleotides changed by the introduced mutations are indicated.

was subjected to RNase H digestion in the presence of DNA oligonucleotides complementary to various segments of the G or AG polarity HDV sequences (Fig. 5C; data not shown). As expected, cleavage of the full-length AG103 RNA (Fig. 5C, lanes 2–5) or its 5' half (AG103s RNA; Fig. 5C, lanes 7–10) was observed with complementary DNAs (a, b, c, and d), and its efficiency correlated with the predicted accessibility of the RNA template to DNA oligo hybridization (Fig. 5D). In the context of the full-length AG103 RNA, only DNAs hybridizing near the ends of the template (a and d) promote cleavage (Fig. 5C, lanes 2 and 5), whereas the ones complementary to sequences buried in a stable double-stranded structure (b and c) do not have an effect (Fig. 5C, lanes 3 and 4). In the mostly single-stranded AG103s RNA, oligos a and b result in a complete RNase H digestion (Fig. 5C, lanes 7 and 8), whereas DNA c promotes only a partial cleavage because its target sequence forms a hairpin structure (Fig. 5C, lane 9). The same DNAs do not promote cleav-

age of genomic polarity RNA (data not shown). Under the same conditions, NE103 RNA product is cleaved by RNase H with DNAs a and b (Fig. 5C, lanes 12 and 13). DNAs c and d do not induce any cleavage of NE RNA product because the region complementary to DNA c forms a stable RNA duplex with the newly synthesized RNA, whereas the region complementary to DNA d is no longer present in the NE product (Fig. 5C, lanes 14 and 15). Although digestion of the AG103s RNA in the presence of a and b DNAs generates two labeled products that correspond to its 5' and 3' segments (Fig. 5C, lanes 7 and 8), only the 3' segments transcribed by pol II are detected upon the analogous digestion of NE product (Fig. 5C, lanes 12 and 13).

These results demonstrate that the NE RNA product represents a chimeric molecule in which a portion of AG RNA template corresponding to the 5' half of the hairpin structure shown in Figures 4A and 5D is covalently linked to an ~40-nt segment of the pol II-transcribed G polarity RNA. This chimeric structure of

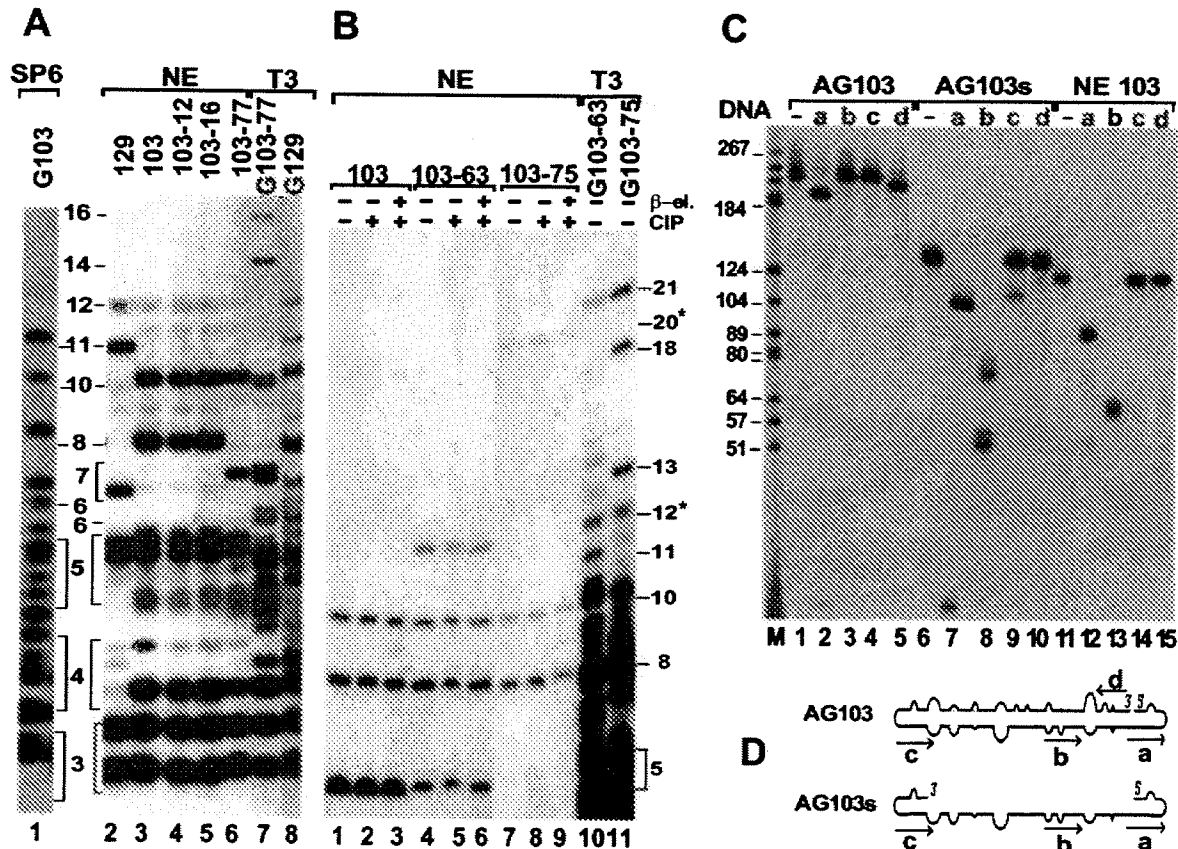


FIGURE 5. Direct RNase analysis of NE RNA transcription products. RNA products were gel purified and subjected to RNase T1 (A and B) or RNase H (C) digestion, and the resulting fragments were resolved in a 25% (A and B) or 7% (C) polyacrylamide/8 M urea gel. **A:** RNase T1 oligonucleotide patterns of NE RNA products 129, 103, 103-12, 103-16, and 103-77 (lanes 2-6) are compared with patterns of SP6-transcribed G103 RNA (lane 1), and T3-transcribed G103-77 and G129 RNAs (lanes 7 and 8, respectively). **B:** Prior to RNase T1 digestion, NE RNA products 103 (lanes 1-3), 103-63 (lanes 4-6), and 103-75 (lanes 7-9) were subjected to no treatment (lanes 1, 4, and 7), CIP treatment (lanes 2, 5, and 8), or β -elimination followed by CIP treatment (lanes 3, 6, and 9). For comparison, RNase T1 digestion patterns of T3-transcribed G103-75 and G103-63 RNAs are included (lanes 10 and 11). The sizes (in nucleotides) of RNase T1 digestion fragments are indicated. The 12-mer and 20-mer that represent the 5'-most oligonucleotides in the digestion patterns of NE103-63 and -75, respectively, are indicated by asterisks. For sequences of AG RNA templates and G polarity products see Figure 3. **C:** RNase H digestion of AG103 RNA template (lanes 1-5), its shorter version AG103s (lanes 6-10), and the NE103 RNA product (lanes 11-15) carried out in the absence (lanes 1, 6, and 11) or in the presence of DNA oligonucleotides a, b, c, and d (as indicated). Positions of pBR322xHaeIII DNA marker are indicated (lane M). **D:** Schematic representation of AG103 RNA template and relative positions of complementary DNA oligonucleotides (see Material and Methods for sequence information).

the NE RNA product suggests that pol II cleaves AG RNA template near the terminal hairpin loop and extends the generated 3' end by copying a portion of the lower strand (i.e., the 5' half of the template).

Sequence determination at the template/transcript junction

The site of template/pol II-transcript junction is ambiguous because of the palindromic nature of AG RNA template, which makes the template sequence in the stem region of the hairpin almost identical to the 5'-terminal sequence of the transcript (Figs. 4A,B and 6C). To precisely identify this junction we created two addi-

tional AG templates in which selected guanosine (G) residues were replaced with adenosine (A) or uridine (U). These mutations eliminate RNase T1 sites only in the template and not in the pol II-transcribed segment of the product.

A unique 12-mer in RNase T1 pattern of NE103-63 RNA that represents the 5'-most 32 P-labeled fragment of this product (Fig. 6A, lane 2) is replaced by a 13-mer in NE103-91 RNase T1 pattern (Fig. 6A, lane 3). This change is only possible if AG template is covalently linked to the pol II transcript, as the G-to-A mutation that eliminates the RNase T1 site in the template would have been neutral if transcribed (see Fig. 6C for sequences). Similarly, substitution of the G:C base pair in

Remarkably, despite multiple mutations in the primary sequence, all of the functional AG RNA templates shown above are cleaved and extended at the same unique site near the terminal stem. Because all these templates can be folded into a similar hairpin structure with characteristic internal loops and bulges, we tested the effect of the secondary structure on specificity of the observed transcription *in vitro*. Both RNase and Pb²⁺ cleavage mapping of AG RNA templates was performed to confirm the predicted secondary structure of these RNAs (J. Filipovska and M.M. Konarska, *in prep.*). The site of cleavage/extension is located at position 129 of AG103 RNA template, immediately adjacent to the UA bulge at positions 130–131 (see Fig. 4A for numbering of positions), suggesting a possible role of this structure in the start site selection. To more closely examine the structure/function relationship of the AG HDV RNA templates, we constructed a mutant AG103-20 RNA, in which the UA bulge is deleted and replaced by a 2-nt insertion at position 113, creating a GC bulge at the opposite side of the terminal stem (Fig. 7B). These mutations are less extensive than sequence changes found, for example, in AG103-63 and 103-75 templates, but they significantly alter the local secondary structure of the RNA, affecting the characteristic bulge adjacent to the start site. Consistent with the predicted importance of this bulge, AG103-20 RNA template fails to support transcription in HeLa NE (Fig. 7A, lane 2). Furthermore, AG103-77 RNA, in which the 62-nt terminal stem-loop region (positions 90–152) is inverted and thus present in genomic polarity, also fails to support transcription (Fig. 7A, lane 3). Remarkably, although these two mutant templates have substantially different primary sequences in the 62-nt

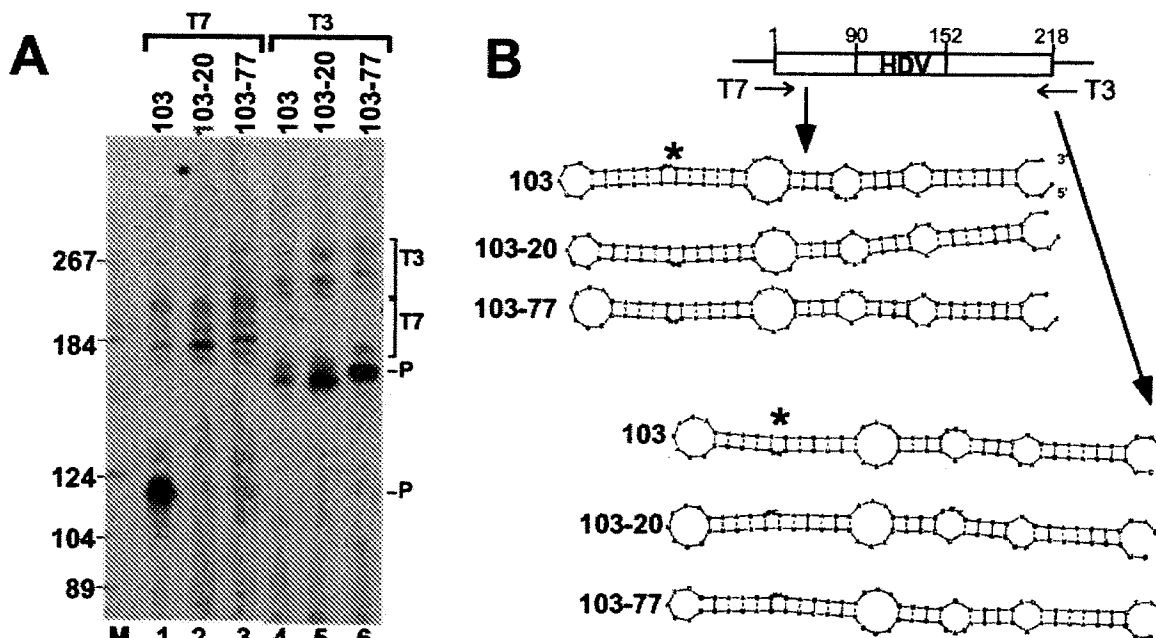


FIGURE 7. Specific secondary structure of HDV RNA, rather than its primary sequence, determines functional templates for transcription. **A:** Two opposite polarities of RNA, synthesized by T7 (lanes 1–3) or T3 (lanes 4–6), were used as templates for transcription in HeLa NE. Products were resolved in a 5% polyacrylamide/8 M urea gel. Positions of the labeled template (T), NE RNA product (P), and pBR322xHaeIII DNA marker (lane M) are indicated. **B:** Schematic representation of a DNA used to synthesize HDV RNA templates from the T7 or T3 promoter and the predicted secondary structure of RNA templates used in **A**. Only the terminal hairpin region (positions 90–152 relative to the AG103 template; see Fig. 3A) is shown. The asterisks indicate positions of the bulge important for template specificity.

terminal stem-loop segment, they both can be folded into structures similar to that of AG103 RNA, but containing a bulge on the opposite side of the terminal stem. In fact, G103 RNA, whose predicted secondary structure resembles that of the inactive AG103-20 and AG103-77 templates, does not support specific transcription in NE (Fig. 7A, lane 4). The faint signal detected in this reaction (Fig. 7A, lane 4) does not correspond to the typical NE product, as indicated by its RNase T1 digestion analysis (data not shown). These results strongly support the importance of a specific secondary structure in the terminal stem-loop of AG HDV RNA in determining its function as a template for pol II transcription. Furthermore, the opposite polarity of AG103-20 and 103-77 RNAs (T3103-20 and 103-77 RNA transcribed by T3 RNA polymerase; see Fig. 7B) efficiently support transcription in NE (Fig. 7A, lanes 5 and 6), confirming the importance of the characteristic terminal stem-loop structure. RNase T1 analysis of the α -amanitin-sensitive NE products of these mutant templates established that transcription starts upstream of the bulge in the terminal stem and proceeds for ~40 nt (data not shown), as observed for reactions using AG103 RNA templates.

Together, these results demonstrate that the 62-nt terminal stem-loop segment is sufficient to determine the specificity of template selection. Although changes

in the primary sequence of this segment are tolerated, its specific secondary structure, and in particular positioning of the bulge adjacent to the transcription start site, is critical for efficient template utilization in this in vitro system.

DISCUSSION

RNA polymerase II responsible for transcription of mRNA in eukaryotes has also been implicated in RNA synthesis from RNA templates during replication of plant viroids and human HDV. Based on the presence of multimeric RNAs detected in infected cells, it is generally accepted that both these classes of RNA pathogens replicate by a rolling-circle mechanism (Chen et al., 1986; Robertson & Branch, 1987). Because of the high complexity of this process that involves multimeric, extensively self-complementary RNA sequences of both polarities, detailed studies of the pol II-mediated RNA replication are very difficult. Here we describe an in vitro system based on HeLa cell NE that supports specific pol II-dependent transcription from HDV RNA templates. Although a full replication cycle cannot be accomplished under these conditions, this system allows for a detailed analysis of both RNA template and protein factor requirements for HDV RNA-templated transcription.

Characterization of HDV RNA-templated transcription in vitro

Using HDV RNA of AG polarity encompassing the region between positions 1109 and 576 of HDV (Fig. 1A, left-hand portion of the rod-like structure) incubated in the presence of HeLa cell NE, we observe specific, template-dependent RNA synthesis. Transcription in this system exhibits a high degree of template specificity. We have tested several other RNAs, including tRNA, snRNAs, 7SL RNA, X-RNA (Konarska & Sharp, 1990), and some other segments of HDV RNA, and failed to detect any transcription products. The observed α -amanitin sensitivity of HDV RNA-templated transcription in both HeLa and PMG NEs demonstrates pol II involvement. Previously, Fu et al. (1993) reported HDV RNA-templated transcription in crude Hep2 nuclear homogenates that was inhibited by α -amanitin and by anti-pol II antibodies. However, more detailed analysis of either RNA products or template requirements was not possible in this system, because long, trimer-length HDV RNAs were used as templates. Similarly, *in vitro* transcription studies of full-length viroid RNA templates were carried out using nuclear homogenates or intact protoplasts of different plant cells as a source of polymerase activity (Mühlbach & Sanger, 1979; Rackwitz et al., 1981; Flores & Semancik, 1982; Semancik & Harper, 1984; Rivera-Bustamante & Semancik, 1989). Again, the specificity of transcription could not be assessed, because these reactions were carried out in the presence of Mn^{2+} , that is, conditions known to reduce polymerase specificity. More recently, Beard et al. (1996) used ~200 nt G polarity HDV RNA corresponding to the right-hand end (opposite to the one used in this study) of the rod-like structure as a template for *in vitro* transcription in HeLa NE. Detection of a specific RNA product in that system implied the presence of a G HDV RNA promoter located in this short RNA segment. However, in the absence of a direct analysis of the RNA product, details of this transcription reaction remain unclear.

To study the mechanism of AG RNA-templated transcription, we analyzed features of HDV RNA that are required for specific template recognition and transcription *in vitro*. Initially, we detected transcription using ~600 nt AG HDV RNA templates. Subsequent experiments demonstrated that as short as ~200 nt RNAs generated by internal and terminal deletions of the initial template are sufficient to support specific RNA synthesis by pol II. This analysis also suggested that a specific structure rather than primary sequence of the RNA template is important for transcription by pol II. Deletions that disrupt the overall hairpin structure of the template interfere with transcription, and this effect is more pronounced if the deletion affects the RNA structure close to the terminal stem-loop of the template (Fig. 2). Notably, this region contains the specific

site where pol II initiates transcription of HDV RNA (Fig. 6), and its secondary structure is important for the template function (Fig. 7). A detailed analysis of NE products demonstrated that pol II precisely copies the RNA template, as individual template mutations are reflected in RNase patterns of the corresponding transcripts. The newly transcribed G RNA is covalently linked to the 5' half of AG RNA, suggesting that transcription in this system does not proceed by *de novo* initiation, but rather, it must involve cleavage of the RNA template followed by extension of the generated 3' end (Fig. 8). A unique site of initiation is located in the stem of the terminal hairpin, immediately adjacent to a bulged dinucleotide structure (Fig. 6; data not shown). The RNA secondary structure surrounding this site is characteristic for all functional mutant templates (Fig. 7).

Transcription proceeds by copying ~40 nt of the 5' half of the template, generating a chimeric template-transcript NE product of ~120 nt (Figs. 6C and 8). It is curious that pol II stops transcription at this specific point. Perhaps this region of HDV RNA contains an RNA sequence/structure arrest signal. Alternatively, limiting concentrations of some protein factors required for transcription may be responsible for this effect.

In typical DNA templated reactions, pol II initiates transcription *de novo*, that is, by extending the new transcript on the 3' OH of the initiating NTP. Selection of the start site and promoter clearance phase of pol II transcription are highly regulated and involve a number of accessory protein factors. During HDV RNA-templated transcription *in vitro*, the initiation event may exhibit distinct requirements and involve instead cleavage of the template that is subsequently used as a

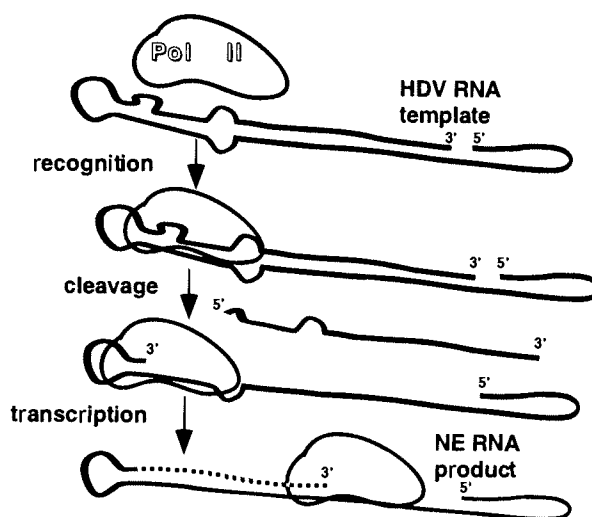


FIGURE 8. A simplified model for AG HDV RNA-templated transcription *in vitro*. The specific hairpin structure of the AG HDV RNA template is recognized by pol II and cleaved adjacent to the characteristic bulge near the terminal loop. The cleaved AG RNA is then used as a template for synthesis of complementary RNA.

primer for transcription. In fact, it has been shown that eukaryotic RNA polymerases I, II, and III, *E. coli*, and vaccinia RNA polymerases in the presence of corresponding elongation factors can endonucleolytically cleave nascent transcripts in paused or arrested ternary complexes in vitro (Uptain et al., 1997, and references therein). During this process, polymerase remains attached to both the DNA template and the nascent transcript, allowing it to efficiently resume RNA synthesis under permissive conditions. Although the physiological role of transcript cleavage by RNA polymerases has not been precisely established, one commonly accepted hypothesis suggests that it serves to release polymerases from transcriptional arrest during elongation of DNA-templated transcription (Reines, 1994). Another proposed role for the transcript cleavage is to increase fidelity of transcription by facilitating removal of misincorporated nucleotides (Erie et al., 1993; Thomas et al., 1998).

Elongation factor-dependent RNA cleavage extension has also been observed in binary complexes of *E. coli* RNA polymerase as well as yeast pol II with CPG79 RNA that encompasses a prokaryotic pause site (Altmann et al., 1994; Johnson & Chamberlin, 1994). The RNA cleavage in these reactions was very slow (~8–20 h) and inefficient, and extension represented the addition of only a few nontemplated nucleotides. In contrast, HDV RNA cleavage extension described here is very fast (5 min) and proceeds efficiently, by precise transcription of the RNA template for up to ~40 nt. Despite the differences in kinetics, these reactions appear to be mechanistically similar. An RNA with a 3'-OH terminus is generated that can be used by the polymerase as a primer for RNA synthesis (Fig. 8). Close examination of CPG79 RNA sequence suggests that it can be folded into a hairpin structure very similar to the terminal hairpin of HDV RNA template used in our system. The importance of RNA hairpin structures in regulation of transcription elongation and termination is well established for prokaryotes (Uptain et al., 1997, and references therein). In eukaryotic systems where transcription elongation and termination are less well characterized, similar RNA secondary structures may also be involved in regulation of transcription (Sharp & Marciniak, 1989; Reeder & Hawley, 1996). Therefore, it is feasible that pol II function during HDV RNA-dependent transcription in vivo is also regulated by a similar hairpin structure.

Analysis of a large number of mutant HDV RNA templates established that RNA-dependent pol II transcription and the precise start site selection in vitro depend on the secondary structure, rather than the primary sequence of the RNA template. In particular, the precise positioning of a 2-nt bulge adjacent to the initiation site appears to specify RNA templates for this reaction; however, other structural elements may also be involved. It will be interesting to identify protein factors

that mediate recognition of these RNA structures, allowing for specific pol II transcription.

Implications for HDV RNA replication in vivo

The rolling circle mechanism for HDV replication during which multimeric intermediate transcripts are synthesized requires intact circular RNA template for transcription (Branch & Robertson, 1984; Chen et al., 1986). Transcription reaction detected in our in vitro system depends on a specific cleavage of the RNA template, implying that it cannot directly represent the initiation step of HDV RNA replication. However, initiation of the rolling circle replication involving a similar mechanism is possible by considering a hypothesis of the RNA template switching. According to this model, pol II would cleave one molecule of HDV RNA and proceed into the elongation phase only after switching to another circular template, using the cleaved template as a primer for initiation of replication. In fact, the use of primers to initiate replication is a common theme among the viruses. For example, tRNAs (Gilboa et al., 1979), capped oligonucleotides cleaved from host mRNAs (Plotch et al., 1981), or a polypeptide primer (Andino et al., 1993) are used by reverse transcriptases, influenza, and poliovirus polymerase, respectively. Utilization of the 3' end of a cleaved HDV RNA molecule as a primer for initiation of HDV replication may represent a similar situation in which RNA structural elements control recognition, specificity of cleavage (initiation) as well as the specificity and efficiency of template switching. HDV and viroids may share a similar pol II-mediated replication mechanism that requires common sequence/structure elements in the RNA (Branch & Robertson, 1984). The template used in this study is derived from the highly conserved, viroid-like region of HDV RNA (Lai, 1995). Identification of the Hepatitis Delta Interacting Protein A (DIPA), a human homolog of HDAG, led to the hypothesis that HDV evolved from a viroid-like RNA that acquired HDAG ORF as a result of pol II switching templates from a viroid ancestor to the cellular mRNA (Brazas & Ganem, 1996). Furthermore, sequence comparison of a number of viroid variants suggested that RNA template switching may frequently occur during viroid replication (Hammond et al., 1989; Koltunow & Rezaian, 1989; Rezaian, 1990; Hernandez et al., 1992; Kofalvi et al., 1997). Notably, stem-loops present at both ends of viroid RNAs are directly implicated in template switching during viroid replication (Semancik et al., 1994; Diener, 1995). Further studies are necessary to determine if a similar template switching may also occur during HDV RNA replication.

Alternatively, it is possible that in contrast to the initiation of HDV transcription observed in vitro, initiation of HDV replication in vivo proceeds by a mechanism that does not require RNA cleavage, but still depends on a specific recognition of the same AG HDV RNA

element for de novo initiation by pol II. In that case, the observed template cleavage could reflect suboptimal conditions of the in vitro reactions, such as the absence of HDAG in HeLa nuclear extracts or limiting concentrations of some cellular factors required for RNA-dependent transcription. Finally, the AG HDV RNA segment may regulate HDV replication in vivo by interacting with pol II machinery and modulating the elongation phase of RNA synthesis rather than its initiation.

The rolling circle mechanism of replication does not strictly require a specific site for initiation of RNA synthesis, because regardless of the initiation site, multimeric transcripts are precisely processed to monomer units by HDV-encoded ribozymes. However, non-specific initiation of replication would imply that pol II recognizes any partially double-stranded RNA as a template for efficient RNA synthesis, which is not observed. In fact, the only two short HDV RNA segments shown to support specific transcription in vitro are localized near the termini of the rod-like structure (Beard et al., 1996; this work). In particular, only the right-hand, and not the left-hand terminal hairpin segment of G polarity served as a template for in vitro transcription (Beard et al., 1996). Similarly, G103 RNA template originating from the left-hand terminal hairpin segment does not support transcription in our system. In contrast, the opposite polarity AG103 RNA is efficiently used in these reactions. Together, these results suggest that pol II-mediated synthesis of both G and AG polarity HDV RNA is controlled by structural RNA elements located at the opposite ends of the rod-like structure. Consistent with this notion, G and AG polarity RNA synthesis during HDV replication in vivo is affected by mutations in separate regions of the genome (Wang et al., 1997; Wu et al., 1997).

In both HDV in vitro transcription systems a specific RNA secondary structure appears to be crucial for RNA synthesis by pol II, as specific mutations that alter characteristic elements of that structure significantly affect transcription (Beard et al., 1996; this study). Furthermore, the same mutations also affect HDV RNA replication in vivo (Beard et al., 1996; J. Filipovska & M.M. Konarska, in prep.). Further studies are necessary to determine the importance of individual stem/bulge elements in directing pol II transcription and involvement of pol II auxiliary factors in this process. The established in vitro systems provide convenient models to study detailed molecular mechanisms underlying pol II-mediated RNA-templated RNA synthesis.

MATERIALS AND METHODS

Plasmids and construction of mutants

The pSG200 plasmid used for synthesis of AG200 RNA template contains *SmaI* fragment of HDV cDNA between positions 481 and 1109 (Wang et al., 1986), cloned into pSKII⁺

vector. *NcoI* cut pSG200 was used for T3 transcription to generate AG200 RNA containing HDV sequences from positions 576 to 1109. As compared to pSG200, pSG128 contains a 183-nt deletion between HDV positions 481 and 664, and a 198-nt deletion between HDV positions 912 and 1109. All plasmids were linearized with *EcoRI* for synthesis of AG RNA templates. As compared to pSG128, pSG130 contains a 28-nt deletion between HDV positions 863 and 890, pSG131 contains a 26-nt deletion between positions 693 to 718, pSG132 contains a 33-nt deletion between HDV positions 718 and 768, and pSG138 contains a 31-nt deletion between HDV positions 822 and 858. pSG129 contains both deletions described for pSG131 and pSG130, and pHS103 contains both deletions described for pSG132 and pSG138.

The mutated versions of the pHS103 used for RNase mapping experiments were constructed using a PCR-based approach, except for pHS103-77 mutant, in which *TaqI* fragment of HDV cDNA, encompassing the terminal loop of the template, was introduced in an opposite orientation by *TaqI* digestion and religation. All sequences were confirmed by sequencing.

RNA templates for HeLa NE transcription

RNA templates were prepared from linearized plasmids using T3 or T7 polymerase. The RNA was incubated in the folding buffer (0.5 M NaCl, 50 mM CAPSO, pH 8.6 at 60 °C, 2 mM MgCl₂, and 0.1 mM EDTA, pH 8.0) for 2 h at 60 °C, ethanol precipitated, resuspended in 25 mM Tris-glycine/25% glycerol, and resolved in a 5% nondenaturing polyacrylamide gel in 50 mM Tris-glycine, pH 8.8. The RNA visualized by UV shadowing was eluted in 0.5 M NH₄OAc, 10 mM MgCl₂, 0.1 mM EDTA, 0.1% SDS buffer and ethanol precipitated. RNA was dissolved in H₂O and stored at -20 °C.

Transcription reactions in HeLa NE

Nuclear extracts from HeLa or PMG cells were prepared as described by Dignam et al. (1983). Optimal conditions for NE transcription with regard to temperature, NE, NTP, Mg²⁺, and salt concentration were determined for both AG200 and AG103 templates and shown to be in the range typical of RNA pol II reactions. Transcription reaction (15 µL) typically contained 5–7 µL of NE (protein concentration ~10 µg/µL) 12 mM HEPES, pH 7.9, 12% glycerol, 60 mM KCl, 0.12 mM EDTA, 0.5 mM DTT, 8 mM MgCl₂, 6 mM of three NTPs, and 0.4 µM (800 Ci/mmol, New England Nuclear) [³²P]-α-NTP. The ³²P-labeled nucleotide was GTP, unless otherwise specified. Approximately 600 fmol of the RNA template was added (although in many cases, titration of the RNA template was used to determine the optimal concentration). In control, DNA-templated reactions, 125 ng (60 pmol) *SmaI*-digested pSmaF DNA (Weil et al., 1979) was used to generate Ad2 MLP RNA. Reactions were incubated for 1 h at 30 °C, RNA products were phenol extracted, ethanol precipitated, and resolved in 5 or 7% polyacrylamide/8 M urea gels.

RNase T1 and RNase H digestion of transcription products

RNA products of T3, SP6, or NE transcription reactions carried out in the presence of α-³²P-GTP unless indicated other-

wise, were gel purified and eluted in the absence of $MgCl_2$ to destabilize secondary structures that interfere with RNase digestion. Eluted RNA was either directly digested with RNase or subjected to Calf Intestinal Phosphatase (CIP) treatment [1 h at 60°C with 1 U AP (Boehringer)] or β -elimination treatment (Muthukrishnan et al., 1975) prior to digestion. For RNase T1 mapping, RNA was dissolved in 10 μ L H_2O , denatured for 10 min at 95°C, and immediately placed on ice. RNase T1 and buffer were then added to a final concentration of 50 mM Tris-HCl, pH 7.7, 0.2 μ g/ μ L yeast tRNA, and 2 U/ μ L RNase T1 (Calbiochem), and the reaction was incubated for 2 h at 50°C. Digestion products were dried, dissolved in 90% deionized formamide, 1 mM EDTA, 0.05% xylene cyanol, and resolved in a 25% polyacrylamide/8 M urea gel. The sequence of the SP6 transcript (SP6 G103-63) complementary to the 5' half of AG103-63 RNA is as follows: 5'-GAAGAAA TCTCTCTAGATTCCGAUAGAGAAUCGAGAGAAAAGUGG CUCUCCCUUGAUCCCCAGGUCGGACCGCGAGGAGGU GGAGAUGCCAUGCCGACCCGAAGAGGAAAGAACUCGA GGGGGGGCCCGGUACCCAAUUCGCCC-3'. For RNase H mapping, annealing was performed in 6.6 μ L containing 50 mM Tris-HCl, pH 8.3, 10 mM DTT, 60 mM NaCl, and 1 μ g DNA oligonucleotide. The sample was denatured for 5 min at 95°C, cooled to 37°C, and placed on ice. The annealed products were then digested with RNase H (0.3 U, Boehringer) in the presence of 4 mM $MnCl_2$ for 30 min at 37°C. The products were phenol extracted, ethanol precipitated, and resolved in a 7% polyacrylamide/8 M urea gel. Sequences of the used DNA oligonucleotides are as follows: (a) 5'-CTCGAGGGGG GGCCCGGT-3'; (b) 5'-TGGAGATGCCATGCCGA-3'; (c) 5'-CGCGATTAGGTGACACTATAGAAGAATCGAGAGAA-3'; (d) 5'-CCGAATTCCATCCTTTC-3'. RNA secondary structures are presented as predicted using the Mfold program by Zuker and Turner at <http://mfold2.wustl.edu/~mfold/rna/form1-2.3.cgi>. The lowest energy structure for each mutant RNA template at 30°C is shown.

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